TITLE OF THE INVENTION

COUMERMYCIN ANALOGS
AS CHEMICAL DIMERIZERS OF CHIMERIC PROTEINS

FIELD OF THE INVENTION

The present invention is directed to coumermycin derivatives useful for promoting the dimerization of chimeric signaling, intracellular proteins.

BACKGROUND OF THE INVENTION

Signal transduction is the process by which extracellular molecules influence intracellular events. A cellular response generally involves multiple signal transduction cascades that operate in concert to elicit a specific biological response. Activities of signal transduction pathways can be regulated through the use of biological molecules, i.e. protein kinases. While many of the proteins in these pathways are known, little is understood about the relationship between particular signal transduction pathways.

In U.S. Patent No. 5,830,462, issued November 3, 1998; and U.S. Patent No. 5,834,266, issued November 10, 1998, both to Crabtree *et al* (both incorporated herein by reference hereto), a general procedure for the regulation of dimerization and oligomerization of intracellular proteins as biological, control mechanisms is suggested. Crabtree further teaches that many signaling pathways originate with the binding of extracellular ligands to cell surface receptors, and receptor dimerization can lead to transphosphorylation and the recruitment of proteins that continue the signal transduction cascade.

In "Activation of the Raf-1 Kinase Cascade by Coumermycin Induced Dimerization," Farrar, M. A., Alberola-Ila, J. and Perlmutter, R. M., Nature, Vol. 383, pp. 178-181, Sept 12, 1996 (incorporated herein by reference hereto), the carboxy terminus of Raf-1 serine/threonine kinase was covalently attached to the amino terminus of the B subunit of bacterial DNA gyrase (GyrB). *Streptomyces* derived natural products, coumermycin and novobiocin, are known to bind GyrB with strong affinity. The region that binds the protein is known to be an oligosaccharide (also known as noviose sugar) and the attached coumarin, a common element between the 2 structures. The stoichiometry of binding between GyrB and drug is 2:1 for coumermycin and 1:1 for novobiocin.

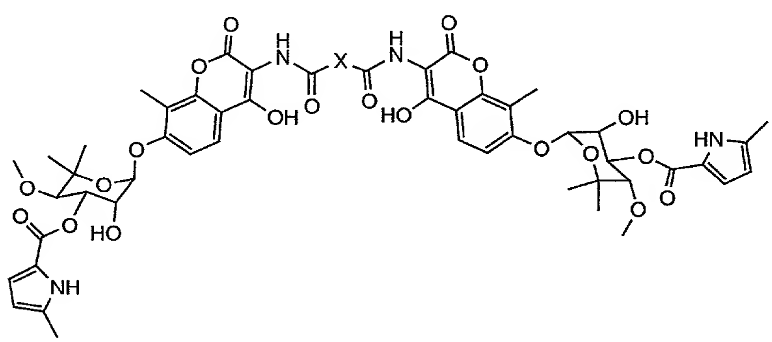
Farrar *et al* suggests adding coumermycin to the chimeric Raf-GyrB fusion protein wherein, coumermycin bound 2 molecules of GyrB that concomitantly brought the 2 Raf kinases into close proximity to one another. This close proximity induced Raf dimerization and led to activation of the Raf kinase cascade.

Furthermore, Farrar *et al* teaches that if a sufficient concentration of novobiocin is added to the coumermycin/GyrB/Raf complex, coumermycin can be displaced from the GyrB active site having similar binding constants and block kinase activation.

Coumermycin is known to have induced dimerization of the tyrosine kinase, Jak2, as well as the transcription factors STAT3, STAT5a, and STAT5b. Unfortunately, it has failed to activate a number of other proteins involved in signal transduction. It is believed that GyrB-Coumermycin-GyrB complex can not orient all kinases and transcript factors into the proper orientation for cross-phosphorylation. A coumermycin analog with a more flexible or longer spacing group will result in the activation of additional signaling proteins, and might have enhanced cellular penetration and potency than coumermycin.

SUMMARY OF THE INVENTION

The present invention is directed to coumermycin analogs of general formula I:



I

or a pharmaceutically acceptable salt or ester thereof,

wherein X is a linking group that connects the two halves of the molecule.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is described herein in detail using the terms defined below unless otherwise specified.

As used herein the term "alkyl" refers to a monovalent alkane (hydrocarbon) derived radical containing from 1 to 18 carbon atoms. It can be straight, branched, or cyclic. Preferred alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, t-butyl, cyclopentyl and cyclohexyl. When the alkyl group is said to be substituted with an alkyl group, this is used interchangeably with "branched alkyl group".

The term "aryl" refers to aromatic rings, e.g., phenyl, substituted phenyl and the like, as well as rings which are fused, e.g., naphthyl, phenanthrenyl and the like. An aryl group thus contains at least one ring having at least 6 atoms, with up to five such rings being present, containing up to 22 atoms therein, with alternating (resonating) double bonds between adjacent carbon atoms or suitable heteroatoms. The preferred aryl groups are phenyl, naphthyl and phenanthrenyl. Aryl groups may likewise be substituted as defined. Preferred substituted aryls include phenyl and naphthyl.

The term "diaryl" refers to two aromatic rings linked together.

The phrase "alkyl with heteroatoms in the chain" refers to alkyl substituents having one or more altered or continuing oxygen, sulfur or nitrogen atom in the chain replacing carbon atoms.

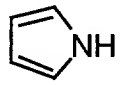
The term "cyclic alkyl" or "cycloalkyl" refers to a specie of alkyl containing from 3 to 18 carbon atoms, without alternating or resonating double bonds between carbon atoms. It may contain from 1 to 4 rings which are fused. Preferred cyclic alkyls are organic substituents having only one ring structure containing alkyl groups therein or having two fused, cyclic ring structures containing alkyl groups therein.

The phrase "combination of alkyl, aryl and heteroaryl" refers to substituents that contain various at least two of the referenced substituents in combination.

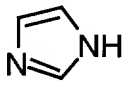
The term "heteroatoms" refers to O, S or N, selected on an independent basis.

The term "heteroaryl" refers to a monocyclic aromatic hydrocarbon group having 5 or 6 ring atoms, or a bicyclic aromatic group having 8 to 10 atoms, containing at least one heteroatom, O, S or N, in which a carbon or nitrogen atom is

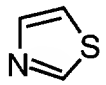
the point of attachment, and in which one or two additional carbon atoms are optionally replaced by a heteroatom selected from O or S, and in which from 1 to 3 additional carbon atoms are optionally replaced by nitrogen heteroatoms, said heteroaryl group being optionally substituted as described herein. Examples of this type are pyrrole, pyridine, oxazole, thiazole and oxazine. Additional nitrogen atoms may be present together with the first nitrogen and oxygen or sulfur, giving, e.g., thiadiazole. Examples include the following:



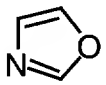
pyrrole (pyrrolyl)



imidazole (imidazolyl)



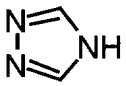
thiazole (thiazolyl)



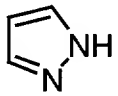
oxazole (oxazolyl)

furan (furyl)

thiophene (thienyl)



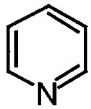
triazole (triazolyl)



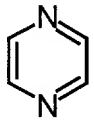
pyrazole (pyrazolyl)

isoxazole (isoxazolyl)

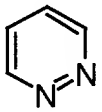
isothiazole (isothiazolyl)



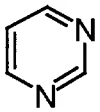
pyridine (pyridinyl)



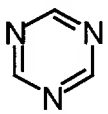
pyrazine (pyrazinyl)



pyridazine (pyridazinyl)

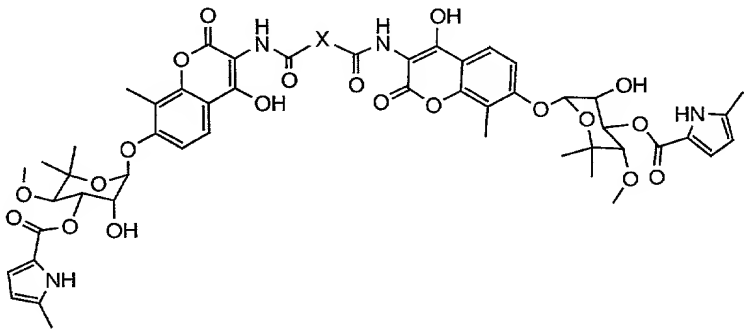


pyrimidine (pyrimidinyl)



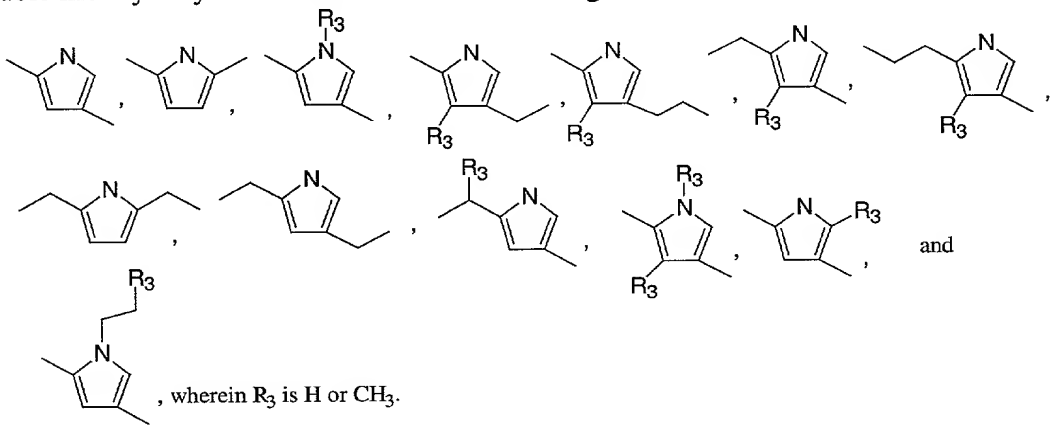
triazine (triazinyl)

The present invention is directed to coumermycin analogs, pharmaceutically-acceptable esters and salts thereof of general formula I:



I

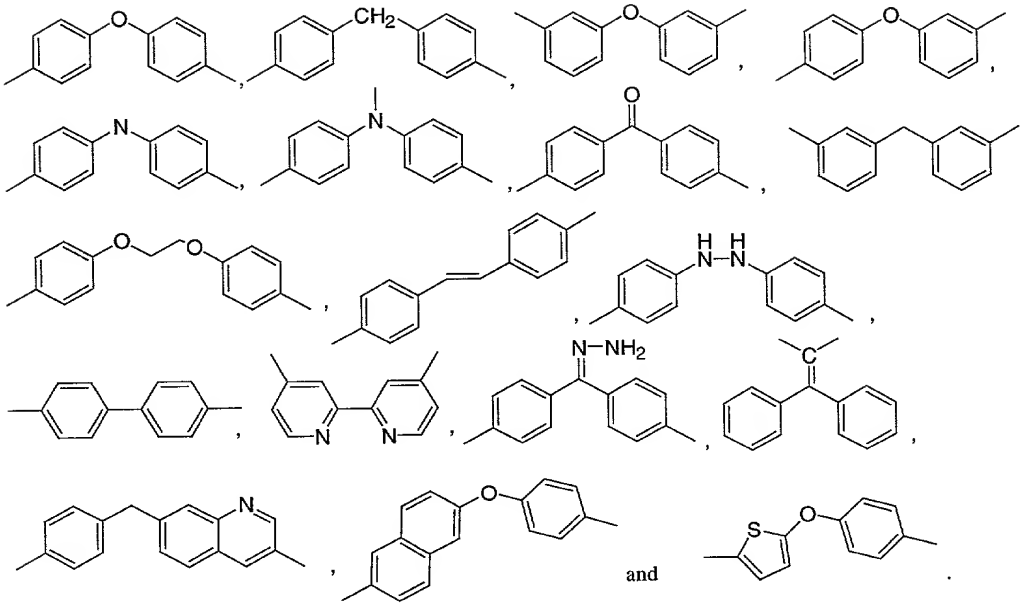
wherein X, a linking group that connects the two halves of the molecule may contain from about 1 to about 54 atoms. The linking group, X, may be selected from alkyl, aryl, diaryl, substituted alkyl, substituted aryl, diaryl, substituted alkyl, alkyl with hereroatoms in the chain, heteroaryl, cyclic and bicyclic alkyl, and a combination of alkyl, aryl and heteroaryl substituents. Optionally, the linking group may also be selected from pyrrole, pyridine, furan, indole, benzofuran, dibenzofuran, thiophene, straight chain alkyl, cycloalkyl, phenyl, diaryl, derivatives and combinations thereof. The pyrrole moiety may be selected from the following:



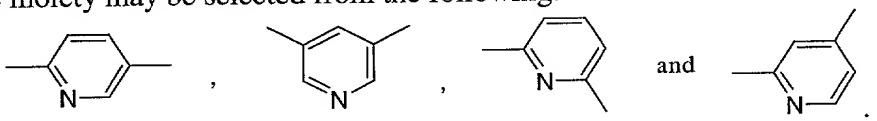
and

, wherein R_3 is H or CH_3.

The diaryl moiety may be selected from the following:

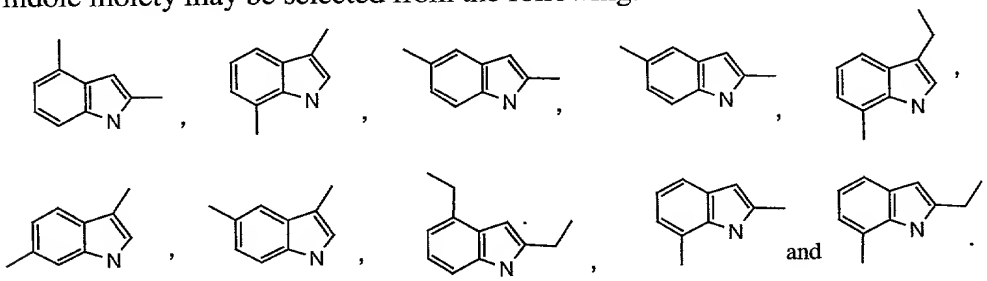


The pyridine moiety may be selected from the following:

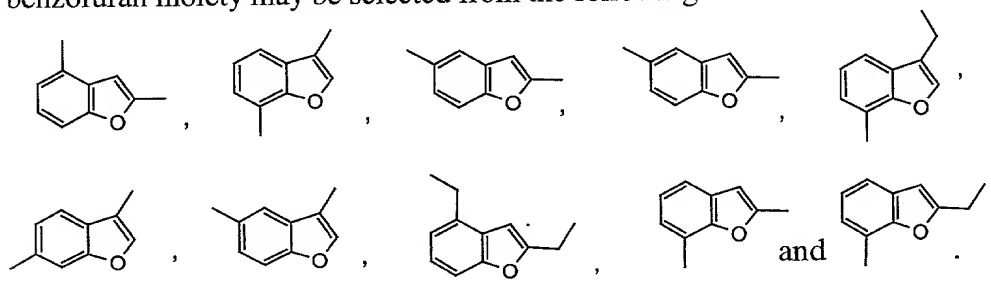


The straight alkyl chain may contain from about 1 to about 18 carbon atoms therein.

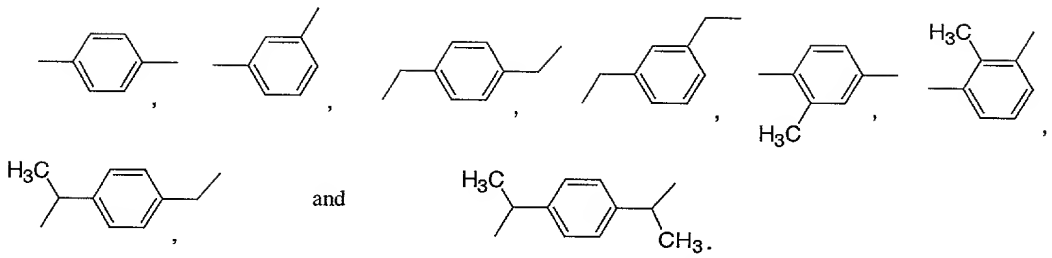
The indole moiety may be selected from the following:



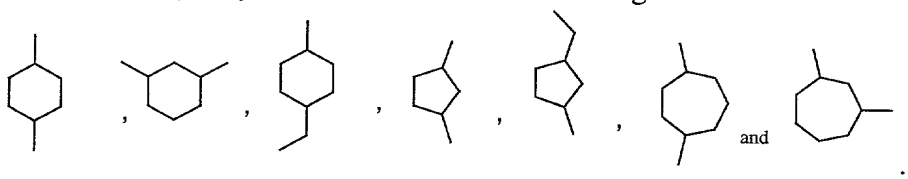
The benzofuran moiety may be selected from the following:



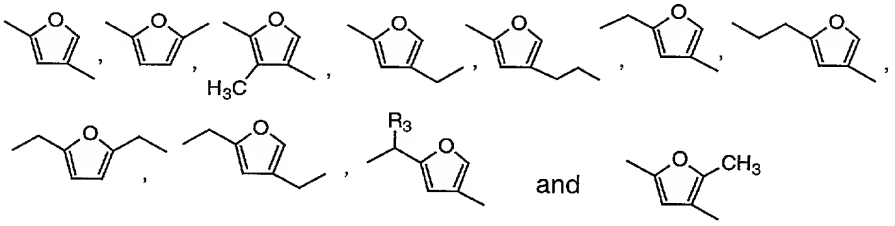
The phenyl moiety may be selected from the following:



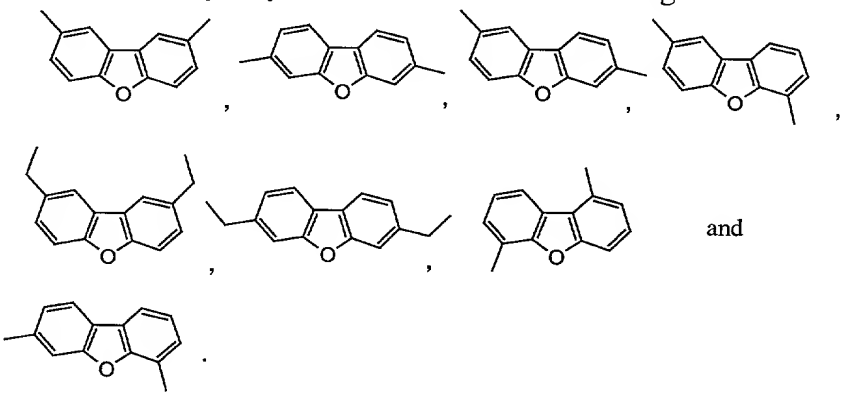
The cycloalkyl moiety may be selected from the following:



The furanyl moiety may be selected from the following:



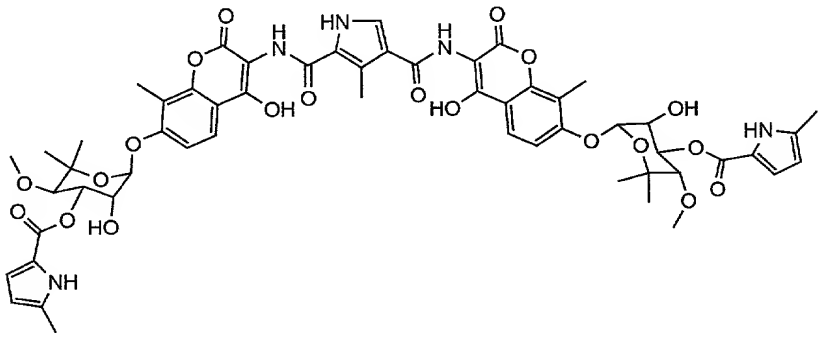
The dibenzofuranyl moiety may be selected from the following:



The analogs are suitable for use as chemical dimerizers oligomerizers of chimeric proteins.

The coumermycin analogs of the present invention are useful as chemical dimerizers of chimeric protein kinases or transcription factors. The analogs are capable of covalently attaching the carboxy terminus of Raf-1 serine/threonine kinase to the amino terminus of the B subunit of bacterial DNA gyrase (GyrB).

Coumermycin is a natural product known to be useful in the inhibition of amino-terminal 24K subdomain of the B subunit of bacterial DNA gyrase (GyrB).

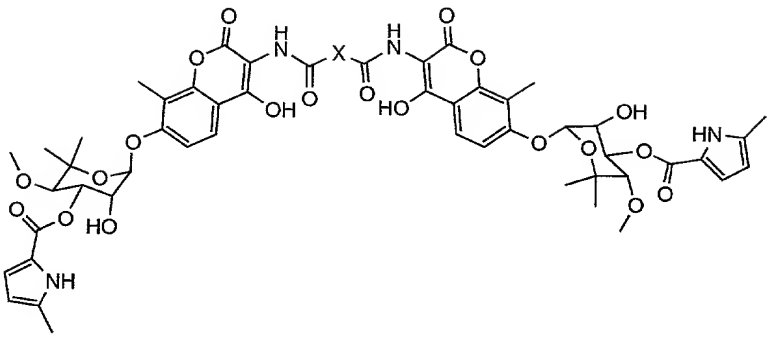


Coumermycin

Analogs of coumermycin may be prepared in accordance with the chemical synthesis sequence from commercially available materials, as presented herein.

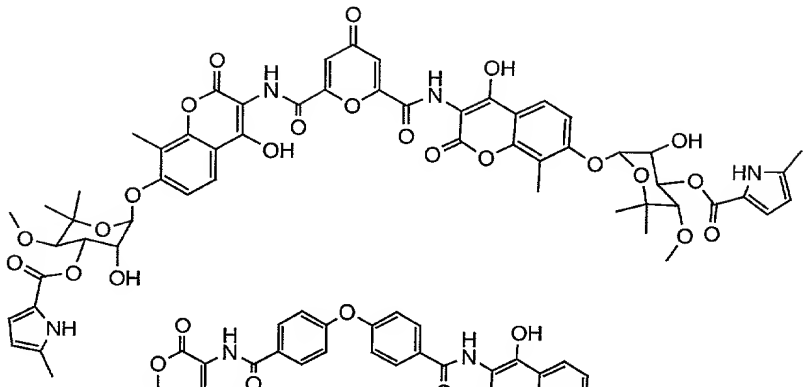
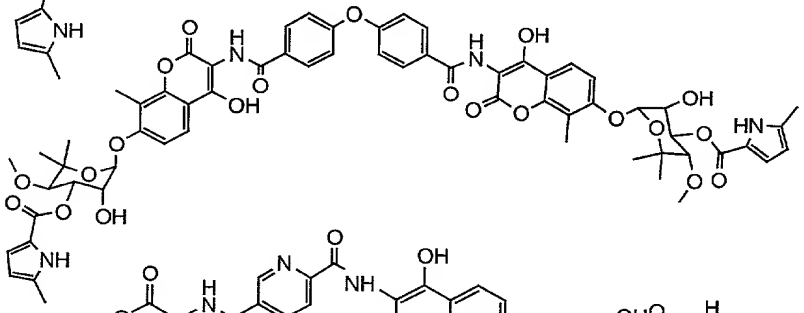
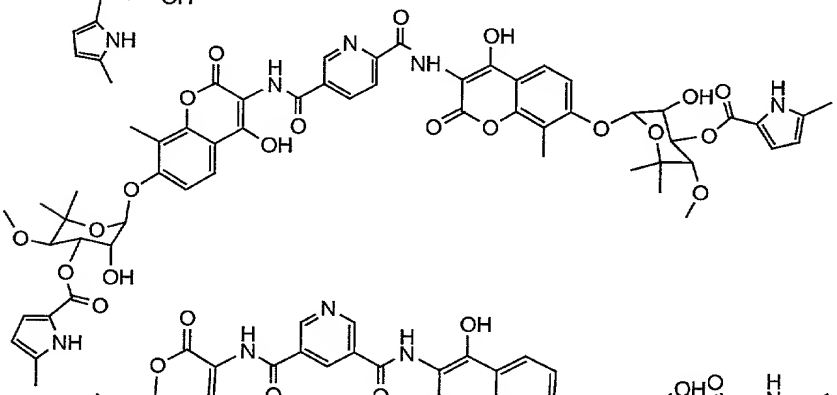
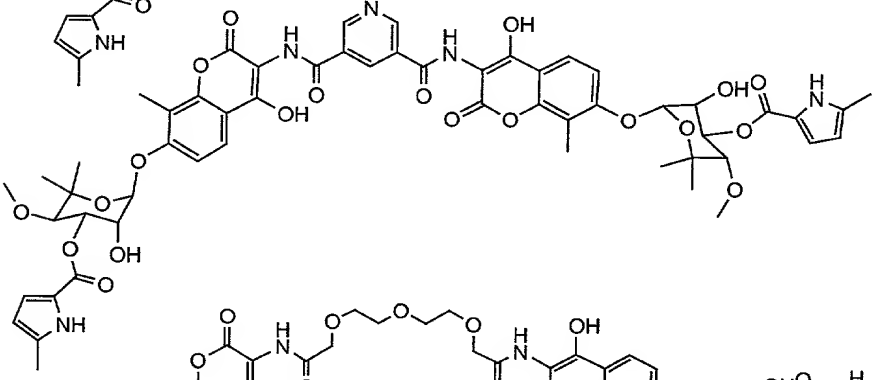
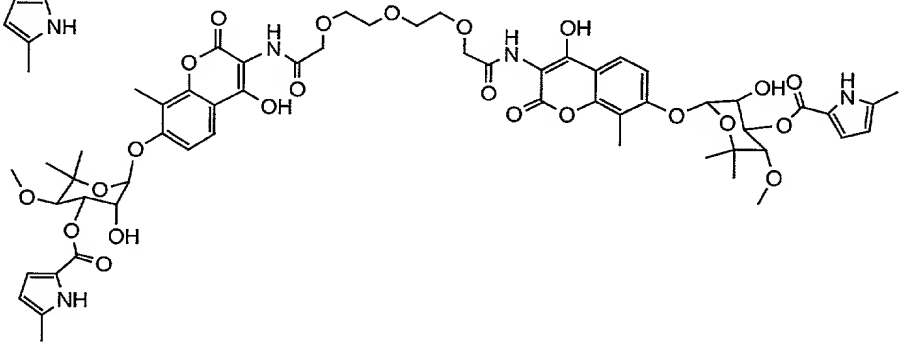
In the present invention, organic molecules regulate signal transduction pathways by activities, among others, proteins or transcription factors. A natural molecule, coumermycin, simultaneously binds two (2) molecules of a B subunit of bacterial DNA gyrase (GyrB) which are, in turn, covalently linked to Raf-1 kinase. The binding event brings the 2 chimeric proteins in close proximity to one another and promotes dimerization (or phosphorylation) of the 2 Raf-1 enzymes which activates a signal transduction pathway. A monomeric natural product, novobiocin, binds just one of these GyrB-Raf-1 chimers and prevents (or turns off) the signaling cascade. Unfortunately the coumermycin/GyrB system can activate only a select few kinase systems. It is, therefore necessary to prepare coumermycin analogs that will induce cross-phosphorylation in a more general manner.

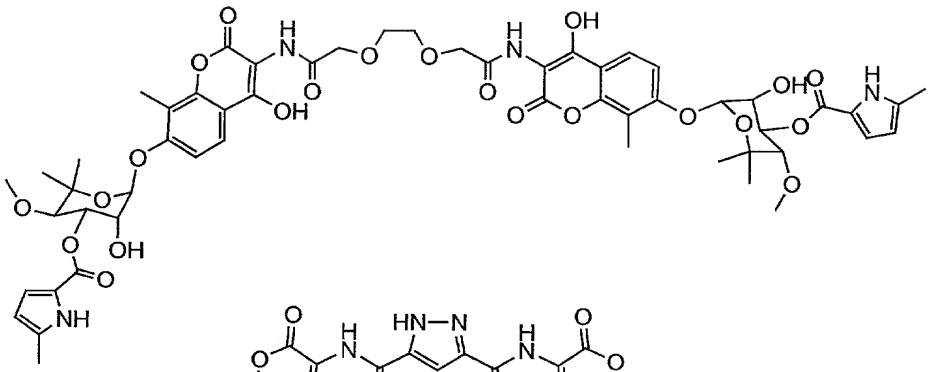
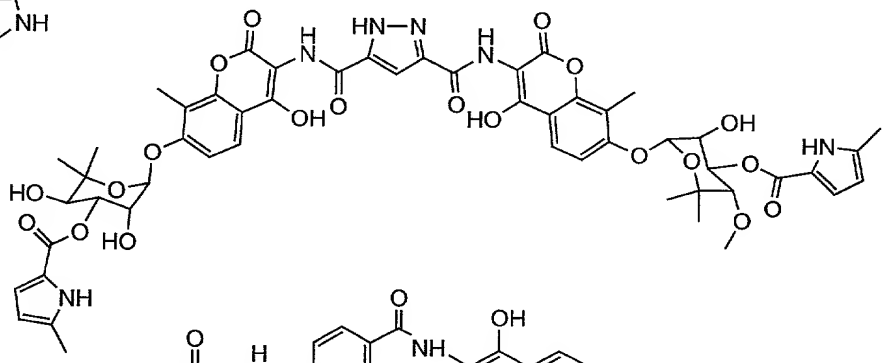
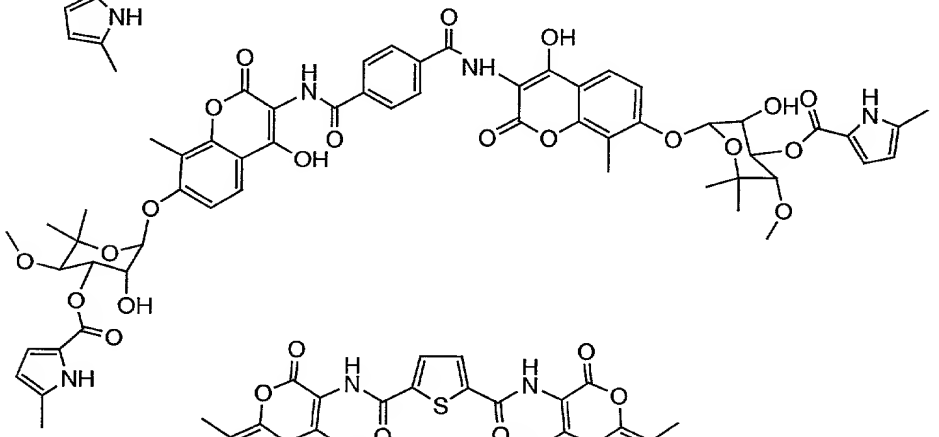
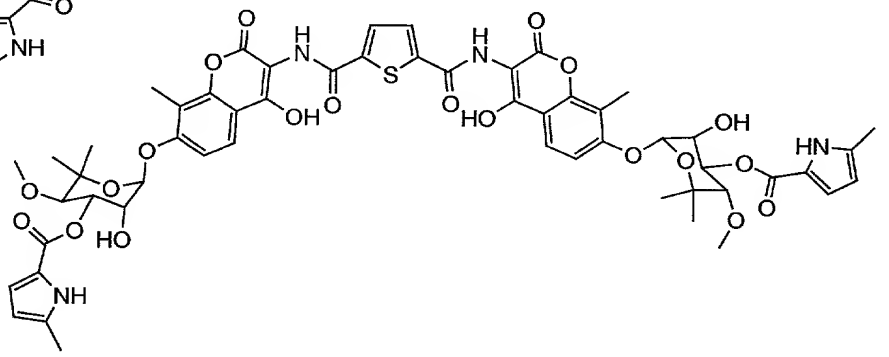
The present invention is further directed to a method of chemically dimerizing chimeric proteins utilizing a coumermycin analog of general formula I:



wherein X is a linking group selected from straight, branched and cyclic alkyl, aryl, diaryl, substituted aryl, diaryl, substituted alkyl, alkyl with heteroatoms in the chain, heteroaryl, a combination of alkyl, aryl, heteroaryl, pyridine, furan, indole, benzofuran, dibenzofuran, thiophene, straight chain alkyl, cycloalkyl, phenyl, diaryl and combinations thereof.

Examples of coumermycin analogs may be selected from the following

Compound No.	Structure
1	
2	
3	
4	
5	

Compound No.	Structure
6	
7	
8	
9	

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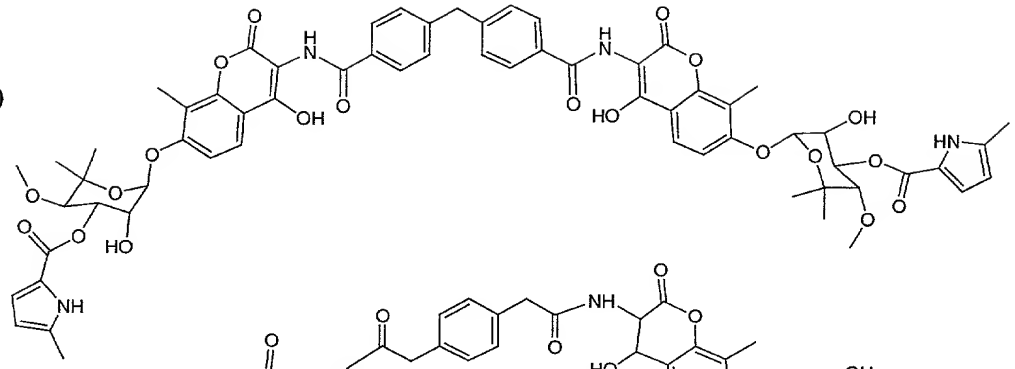

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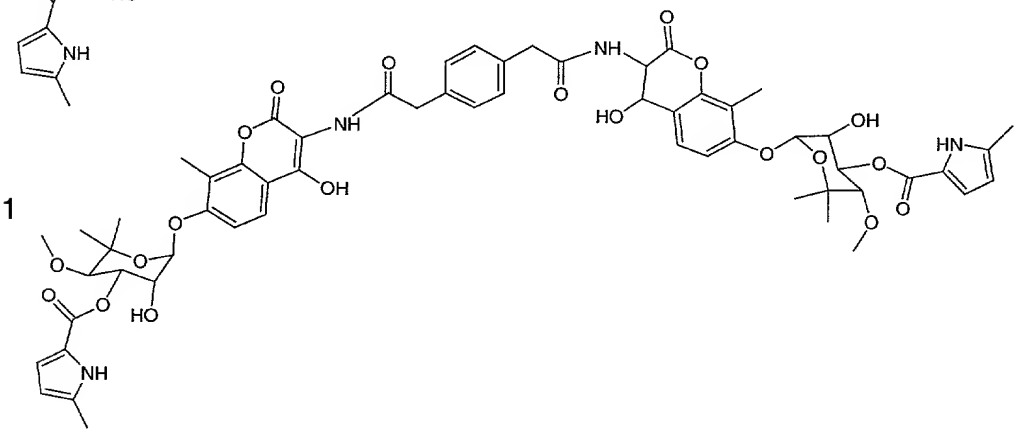
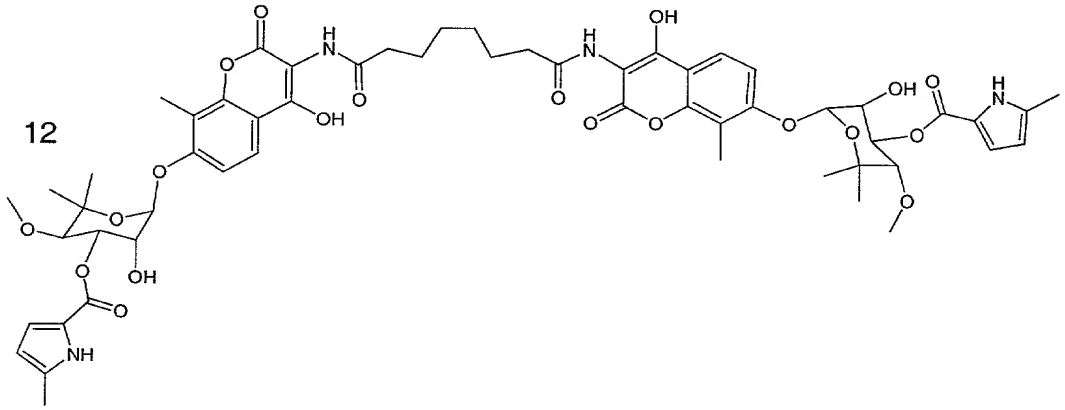


12



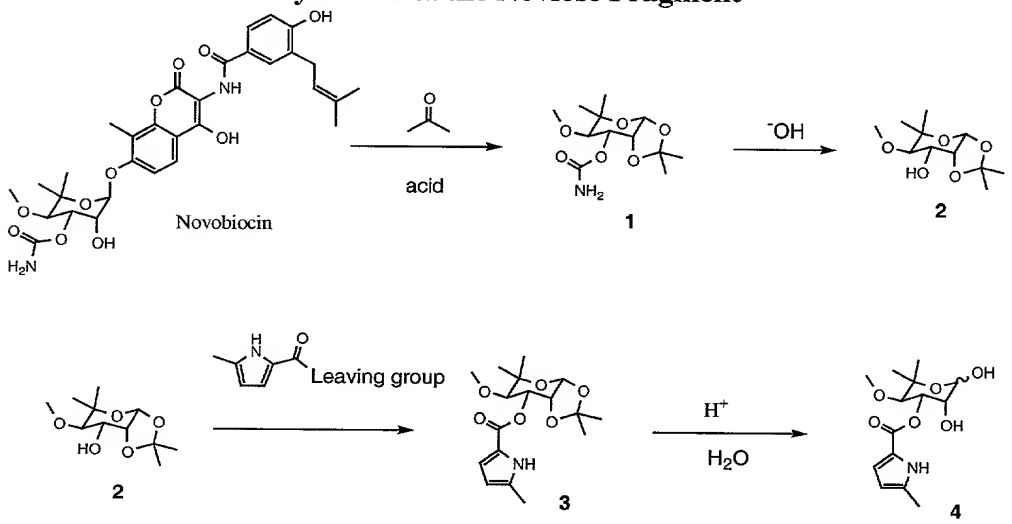
Synthesis Schemes

The compounds of this invention can be synthesized by methods known in the art. As illustrated herein, the compounds can be synthesized in accordance with Synthesis Schemes I through V.

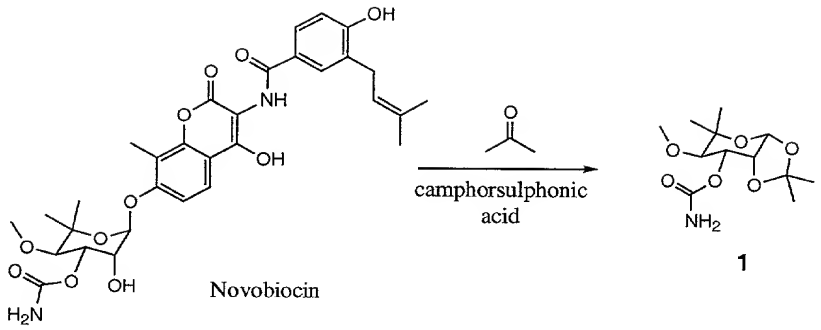
Those skilled in the art will appreciate other methods thereof after reviewing the following:

SCHEME I

Synthesis of the Noviose Fragment



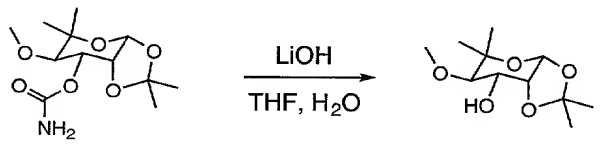
A protected version of the noviose sugar (**1**) is prepared by exposing commercially available novobiocin to a Lewis acid or protic acid, preferably a strong protic acid, more preferably toluenesulfonic acid in the presence of acetone. Hydrolysis of the C-3' carbamate moiety is accomplished by stirring with hydroxide, preferably lithium hydroxide, to give **2**. The C-3' position of the sugar was acylated by exposing **2** to a derivative of 5-methyl pyrrole-2-carboxylic acid where the carboxylic acid is activated for acylation. Preferably the pyrrole is activated as an anhydride (see compound **7** in Scheme II) and preferably an acylation catalyst is used, more preferably the acylation catalyst is tributyl phosphine. Protected sugar **3** is converted to the reducing sugar **4** by exposure to acid, preferably trifluoroacetic acid.



Novobiocin

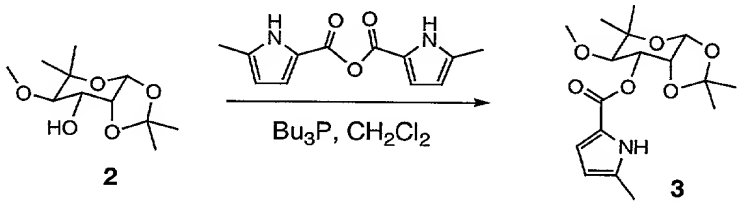
1

A stirring mixture of novobiocin (12.0 g), *p*-toluenesulfonic acid (3.6 g) in 100 mL anhydrous acetone was heated to 48 °C. A white precipitate formed during course of the reaction. After six hours, the cloudy white solution was cooled to room temperature and the acetone was removed *in vacuo*. The resulting solid was purified by column chromatography (basic alumina, 100% ethyl acetate) to give 3.2 g of the protected noviose sugar **1**. Mass spectra (MS) (ESI) $m/z = 298$ ($M+Na^+$).



1 **2**

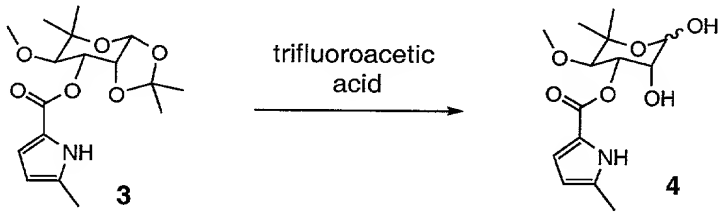
Sugar acetonide **1** (2.5 g) was dissolved in 30 mL methanol and 30 mL THF (tetrahydrofuran). Lithium hydroxide hydrate (2.9 g) was dissolved in 30ml of water and added to the stirring solution. The solution was warmed to 40 °C and stirred overnight. The product **2** was extracted with water and ethyl acetate (3X). No purification was necessary. MS (ESI) $m/z = 255$ ($M+Na^+$).



2 **3**

Noviose sugar (0.82 g) **2** and pyrrole anhydride **7** (1.6 g) were dissolved in 50 mL of dry methylene chloride and stirred at room temperature under nitrogen.

Tributylphosphine (0.77 g) was added and the solution was stirred for 60 hours. The solution was added to 30 mL water and extracted twice with methylene chloride. The organic layers were combined, dried over magnesium sulfate, filtered, and dried *in vacuo*. The crude sugar was purified by silica gel chromatography (20% → 25% → 33% ethyl acetate in hexane) to provide the acylated noviose **3** (0.99 g). MS (ESI) *m/z* = 362 ($M+Na^+$).

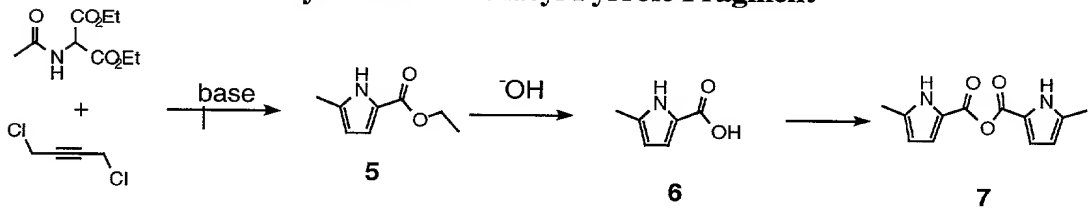


3 **4**

The protected noviose (513 mg) was dissolved in a 4:1 mixture of trifluoroacetic acid (TFA):water (5 mL) and stirred at room temperature for five minutes. The solvents were removed *in vacuo* and the resulting solid was filtered through silica gel (100% ethyl acetate). The purified reducing sugar **4** was obtained as a mixture of anomers (381 mg). MS (ESI) *m/z* = 322 ($M+Na^+$).

SCHEME II

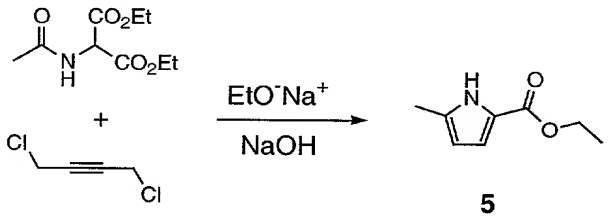
Synthesis of the Acyl Pyrrole Fragment



5 **6** **7**

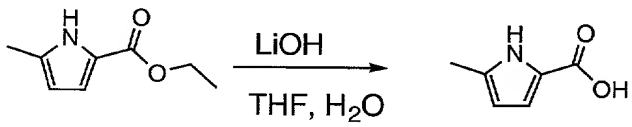
Syntheses of 5-methylpyrrole-2-carboxylic acid and its derivatives are well known in the literature. We found the method of Curran, T. P., Keaney, M. T. *J. Org. Chem.* 61, 9068 (1996) to be preferred. Mixing ethyl acetamidomalonate and 1,4-dichloro-2-butyne in the presence of base, preferably sodium ethoxide, provided pyrrole ester **5**. Hydrolysis in the presence of hydroxide, preferably lithium hydroxide gave the desired carboxylic acid **6**. Activation of the carboxylic acid **6** was

accomplished by exposing the carboxylic acid to a peptide coupling reagent, preferably a carbodiimide, more preferably 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. The anhydride product **7** can be used in Scheme I to acylate the noviose sugar fragment **2**.



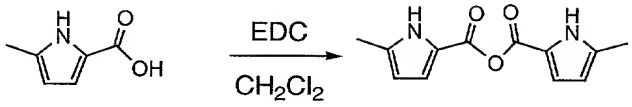
5

The procedure of Curran, T. P., Keaney, M. T. *J. Org. Chem.* 61, 9068 (1996) was followed. Diethyl acetamidomalonate (0.395 g) was added to a solution of sodium ethoxide in ethanol (1.0 M, 9 mL) and the solution was heated to reflux (95 °C to 97 °C). 1,4-dichloro-2-butyne (190 µL) was added and the solution was refluxed for one hour. Additional portions of 1,4-dichloro-2-butyne (190 µL) and sodium ethoxide in ethanol (0.70 mL, 21 wt. %) were added and heating continued for another hour. When complete, the solution was cooled to room temperature and the ethanol was removed *in vacuo*. The brown residue was dissolved in ethyl acetate and extracted water (2X), saturated sodium bicarbonate (3X), 1N HCl (3X), and brine. The organic layer was dried over magnesium sulfate, filtered and evaporated. The resulting solid was purified by column chromatography (silica gel, 10% ethyl acetate in hexane) to give 130 mg of the ethyl ester **5**. MS (ESI) m/z = 154 ($M+H^+$).



5 **6**

The ethyl ester **5** (4.5 g) was dissolved in methanol (50 mL) and THF (50 mL). Lithium hydroxide hydrate (10.0 g) was dissolved in water (50 mL) and added to the stirring solution. The reaction was stirred overnight at 40 °C. The crude product was added to dilute aqueous HCl (pH between 1.5- 3.0) and extracted with ethyl acetate (3X). The organic layers were combined, dried over magnesium sulfate, and filtered and evaporated to give **6** (3.6 g). No purification was necessary.



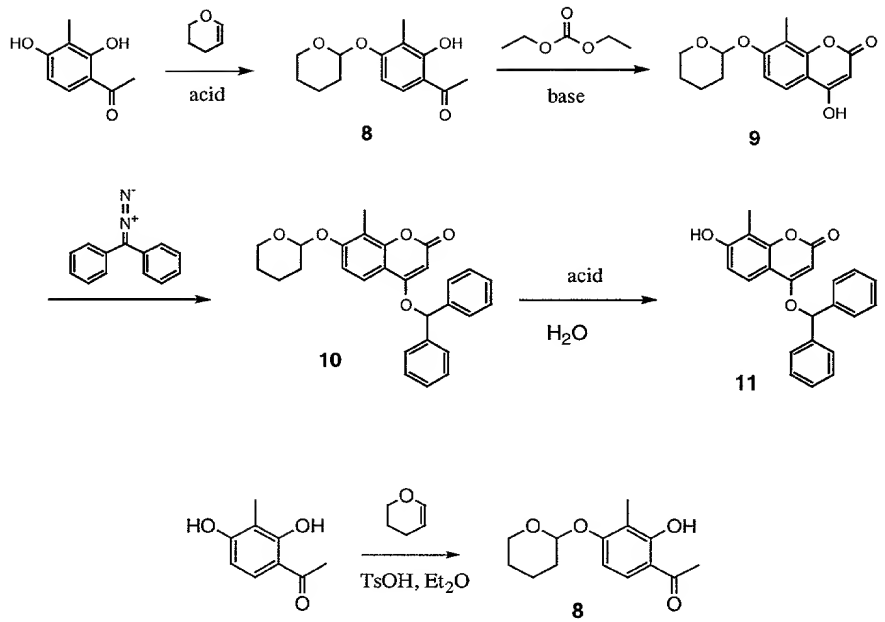
6 **7**

5-methyl-pyrrole-2-carboxylic acid **6** (4.8 g) was dissolved in methylene chloride (150 mL) and stirred at room temperature under nitrogen. 4-methylmorpholine (2.11 mL) was added followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.68 g). After stirring for two hours, the crude material was added to water and extracted with methylene chloride (3X). The organic layers were combined, dried over magnesium sulfate, filtered, and dried *in vacuo*. The product was purified by column chromatography (silica gel, 33% ethyl acetate/ 67% hexane) and provided the desired anhydride **7** (3.65 g). MS (ESI) $m/z = 233$ ($M+H^+$).

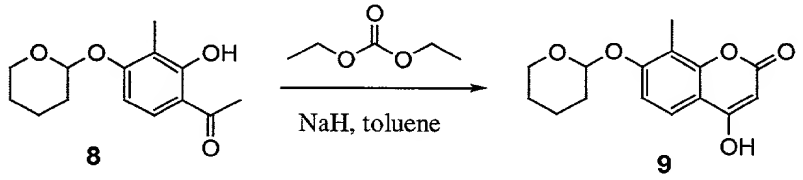
SCHEME III

Synthesis of the Coumarin Fragment

A number of literature procedures exist for the synthesis of the coumarin core of coumermycin. The method of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) and Haesslein, J-L. *et al.* WO 99/35155 is preferred. Commercially available 2,4-dihydroxy-3-methylacetophenone was selectively protected with dihydropyran is the presence of acid, preferably toluenesulfonic acid, to provide **8**. The coumarin fragment **9** was prepared by exposure to an appropriate electrophile, preferably diethyl carbonate, to in the presence of base. Protection of **9** was accomplished addition of diphenyl diazomethane to form **10**. Removal of the tetrahydropyranyl protecting group occurred in the presence of water under acid catalysis to yield compound **11.**

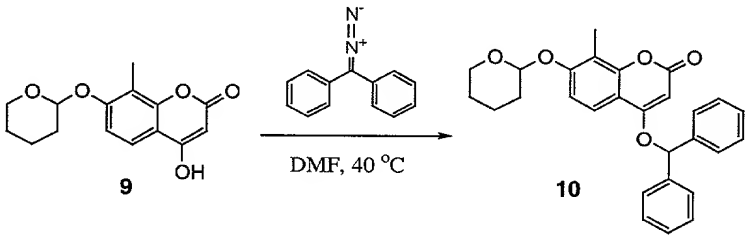


The procedure of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) was followed. Anhydrous diethyl ether (130 mL) was added to 2,4-dihydroxy-3-methylacetophenone (21.52 g). The mixture was stirred under nitrogen and dihydropyran (21.5 mL) was added. The mixture was cooled to 0 °C. Toluenesulfonic acid (118 mg) was added and the reaction was allowed to slowly warm to room temperature. After twelve hours another portion of toluenesulfonic acid (80 mg) was added. The black solution was stirred an additional six hours and then added to a saturated sodium bicarbonate solution. Extraction with ether followed by drying over magnesium sulfate, filtration and solvent removal provided a black solid which was purified by column chromatography (Silica gel, 20% → 25% → 33% ethyl acetate in hexane) to provide the protected acetophenone **8** (15.9 g). MS (ESI) m/z = 251 ($M+H^+$)



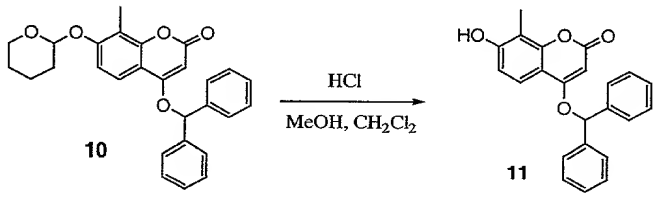
The procedure of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) was followed. Protected acetophenone **8** (15.9 g) was dissolved in toluene (170 mL).

Diethyl carbonate was added and the solution was heated to 90 °C under nitrogen with vigorous stirring. Sodium hydride (60% in mineral oil, 5.87 g) was added portionwise to prevent foaming. Stirred at 90 °C overnight. Cooled to room temperature and an off-white solid was collected after centrifugation. The solid was triturated with ether, 1M NaH_2PO_4, water, acetone, and ether again and the resulting sodium salt of the coumarin was collected as a white solid. The coumarin was dissolved in methanol and acidified with acetic acid. Water was added and the methanol was removed *in vacuo*. The white precipitate was collected by filtration and dried to give the desired coumarin **9**. MS (ESI) $m/z = 277$ ($M+H^+$)



9 10

The procedure of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) was followed. The coumarin **9** (2.85 g) was dissolved in dimethyl formamide (DMF) (13 mL) and warmed to 90 °C under nitrogen. Diphenyl diazomethane (3 g) was dissolved in DMF (13 mL) and added dropwise to the reaction mixture over 150 minutes. After two hours additional diphenyl diazomethane (400 mg) was added. After two more hours diphenyl diazomethane (400 mg) was added again. Stirred an additional 6 hours at 90 °C. The solution was cooled to room temperature and added to water. After extraction with ethyl acetate (3X) the organic layers were combined, dried over magnesium sulfate, filtered and evaporated *in vacuo*. The crude mixture was chromatographed (silica gel, 100% methylene chloride → 1% → 2% → 3% → 4% → 5% → 50% → 100% ethyl acetate in ethyl acetate in methylene chloride) to provide the fully protected coumarin **10** (2.77 g). MS (ESI) $m/z = 443$ ($M+H^+$)

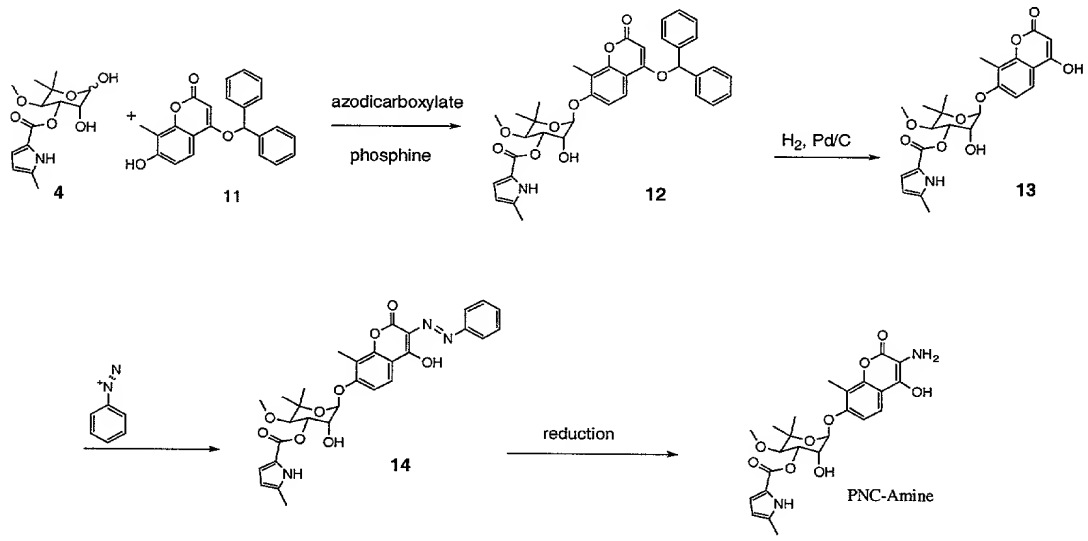


10 11

The procedure of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) was followed. The coumarin **10** (2.77 g) was dissolved in anhydrous methylene chloride (20 mL) and anhydrous methanol (20 mL) and stirred at room temperature under nitrogen. Acetyl chloride (0.31 mL) was slowly dissolved in anhydrous methanol (5 mL) and added to the stirring solution of coumarin. After 90 minutes a white precipitate formed. The mixture was neutralized with aqueous saturated sodium bicarbonate and extracted with methylene chloride. The organic layer was dried over magnesium sulfate, filtered and evaporated. The coumarin **11** was purified by trituration with ethyl acetate. MS (ESI) m/z = 359 ($M+H^+$).

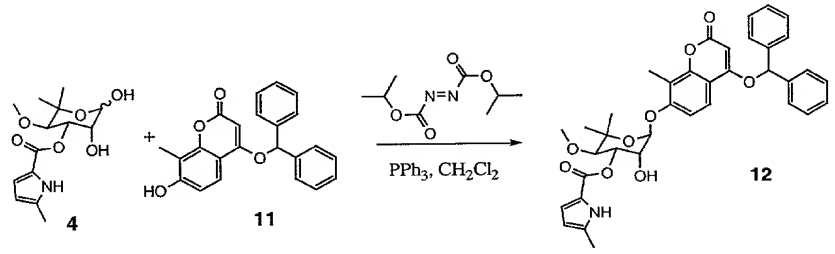
SCHEME IV

Synthesis of PNC-Amine

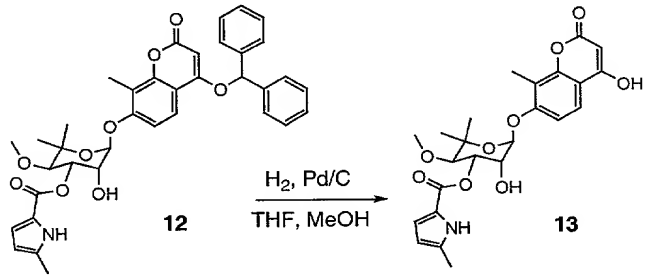


The pyrrole-sugar fragment **4** and the coumarin fragment **11** were coupled to form fragment **12**. Preferably they were coupled using an azodicarboxylate and a phosphine derivative, more preferably they were coupled using diisopropylazodicarboxylate and triphenyl phosphine. The protecting group of **12** was removed by hydrogenolysis, preferably hydrogen gas in the presence of palladium, to provide **13**. Diazotizaton of **13** with diazobenzene provided **14** that was reduced to the **PNC-amine**, (PNC = 3-amino-4-hydroxy-8-methyl-7-[[3-O-[(5-methyl-2-pyrrolyl)carbonyl]noviosyl]oxy]coumarin) preferably with sodium dithionite.

The **PNC-amine** is a late-stage building block that can be readily converted to the natural product coumermycin and many other dimeric coumarin derivatives, including those in the illustrated examples and in the claims section hereof.

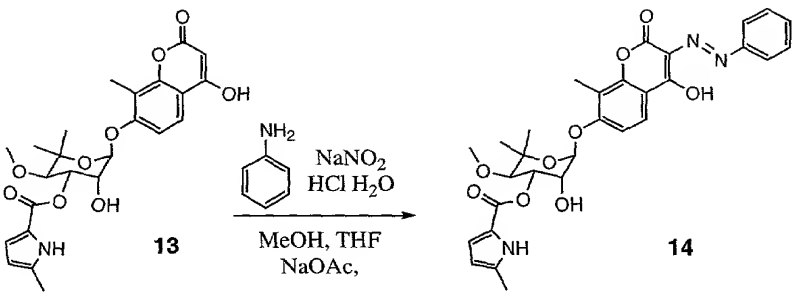


The procedure of Laurin, P. *et al.*, *Bioorg. Med. Chem Lett.* 9, 2079 (1999) was followed. The coumarin **11** (123 mg) and reducing sugar **4** (82.6 mg) were suspended in methylene chloride (5 mL) and stirred at room temperature under nitrogen. Triphenyl phosphine (101 mg) was added followed dropwise addition of diisopropyl azodicarboxylate (0.087 mL in 0.40 mL methylene chloride). After two hours, additional portions of triphenyl phosphine (36 mg) and diisopropyl azodicarboxylate (0.027) were added. Stirred under nitrogen overnight. Worked up by evaporation of methylene chloride *in vacuo*. Purified by repeated chormatography in silica gel (25% $\rightarrow$ 33% $\rightarrow$ 50% ethyl acetate in hexanes) to give the desired α-glycoside **12** (73 mg) and the undesired β-glycoside (32.6 mg). MS (ESI) m/z = 640 ($M+H^+$).

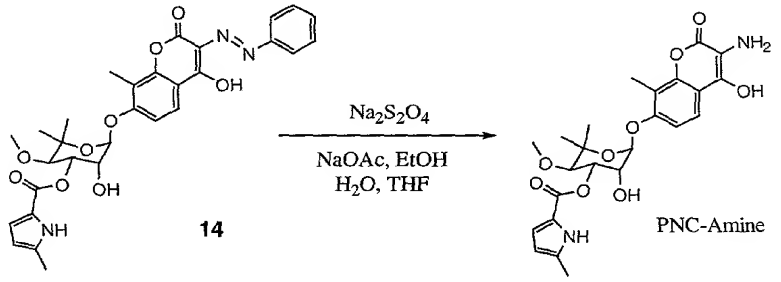


The α-glycoside **12** (73 mg) was dissolved in THF (2.5 mL) and ethanol (2.5 mL). Palladium on Carbon (10%, 14 mg) was added and the solution degassed. One atmosphere of hydrogen gas added and the reaction was stirred overnight. Hydrogen gas was removed *in vacuo* and the solution was filtered though celite. The eluant was

evaporated and the solid **13** was taken crude to the next step. MS (ESI) *m/z* = 474 ($M+H^+$).



Aniline (0.10 mL) was added to a stirring solution of aqueous HCl (1N, 3.84 mL) at 0 °C. Sodium nitrite was dissolved in water (0.35 mL) and added dropwise to the aniline solution. After 10 minutes, an aqueous solution of sodium acetate (405 mg in 0.70 mL water) was added and the resulting diazo benzene was stirred 10 additional minutes at 0 °C. The noviose-coumarin **13** was dissolved in methanol (3 mL) and THF (1 mL) and stirred at room temperature. One equivalent of the diazobenzene solution was added dropwise to the coumarin and the solution turned a deep orange. The product was added to a saturated aqueous ammonium chloride solution and extracted with ethyl acetate (3X). The organic layers were combined, dried over magnesium sulfate and evaporated. The product **14** was taken to the next step without further purification. MS (ESI) *m/z* = 578 ($M+H^+$).

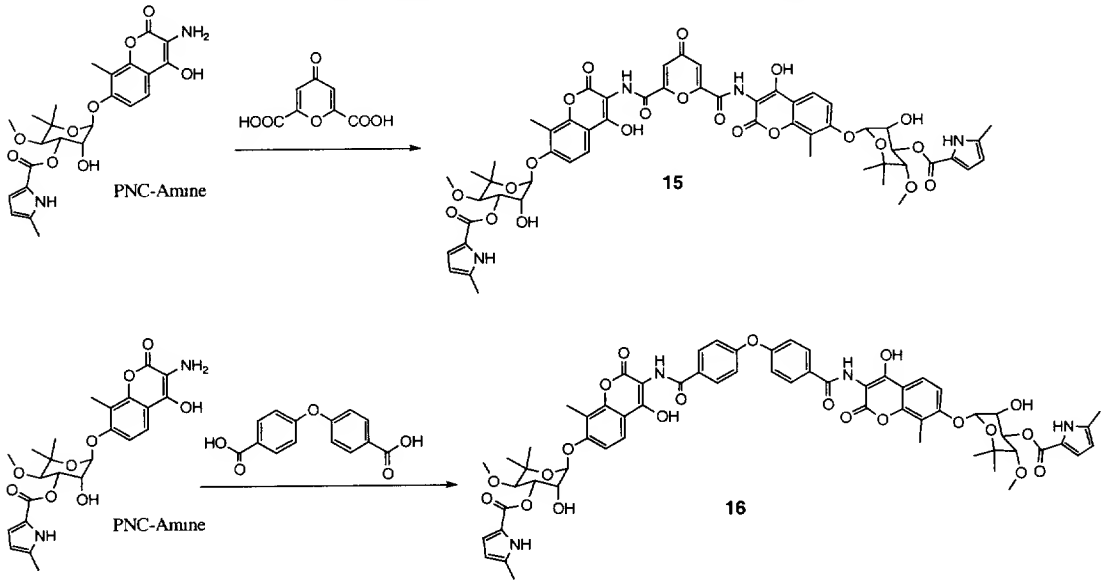


The diazotized coumarin **14** was dissolved in THF (3 mL) and Ethanol (6 mL). The solution was stirred at room temperature and sodium acetate (56 mg dissolved in 1 mL water) was added. Sodium dithionate (80 mg) was then added and the solution turned from dark orange to light yellow. The solvents were then dried *in vacuo* and

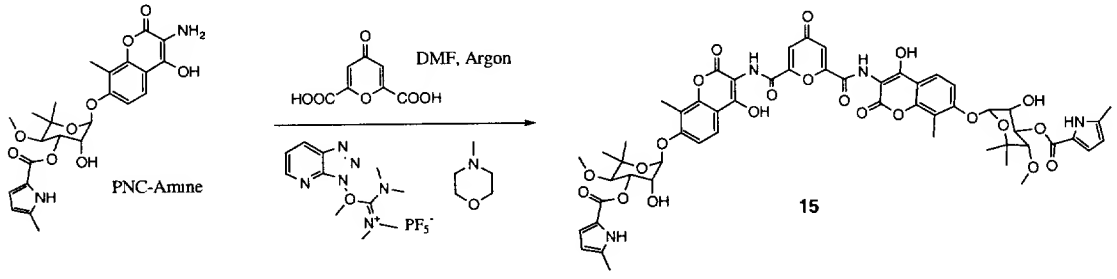
resulting crude **PNC-amine** was purified by silica gel chromoatography. (1:9:90 ammonium hydroxide : methanol : methylene chloride → 2:18:80 → 3:27:70) to give pure PNC-amine (48.1mg). MS (ESI) m/z = 489 ($M+H^+$).

SCHEME V

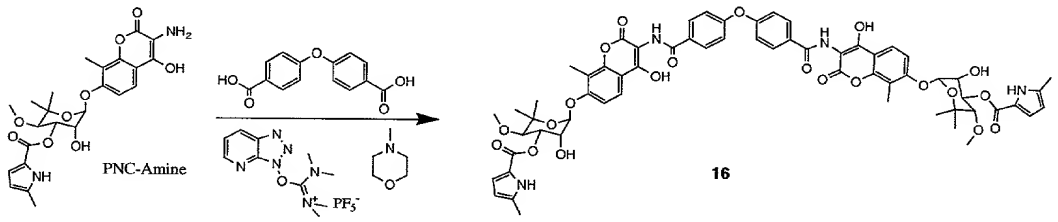
Preparation of Coumermycin Analogs



The coumarin analogs were prepared by the method shown in Scheme V. The **PNC-Amine** was exposed to a dicarboxylic acid or a derivative thereof to yield coumarin derivatives such as **15** and **16**. Preferably, the **PNC-Amine** was exposed to a dicarboxylic acid and a peptide coupling reagent, most preferably the peptide coupling reagent was O-(azabenzotriazole-1-yl) N,N,N',N'-tetramethyluronium hexafluorophosphate).



Chelidonic acid (2.9 mg) and O-(azabenzotriazole-1-yl) N,N,N',N'-tetramethyluronium hexafluorophosphate) (14.2 mg) were dissolved in dry deoxygenated DMF (1 mL) and stirred at room temperature under argon. 4-methylmorpholine (10.0 μL) was added to the reaction. After 15 minutes, **PNC-amine** (21.4 mg) was dissolved in dry deoxygenated DMF (0.4 mL) and added slowly to the activated diacid. The reaction was stirred overnight over argon and after 12 hours, the crude DMF solution was purified by injecting the solution onto the HPLC column where the final product **15** (3.3 mg) was readily purified (RP18, gradient, 90% $H_2O \rightarrow$ 100% acetonitrile). MS (ESI) m/z = 1125 ($M+H^+$).



4,4;-oxybis(benzoic acid) (4.5 mg) and O-(azabenzotriazole-1-yl) N,N,N',N'-tetramethyluronium hexafluorophosphate) (13.2 mg) were dissolved in dry deoxygenated DMF (1 mL) and stirred at room temperature under argon. 4-methylmorpholine (10.5 μL) was added to the reaction. After 15 minutes, **PNC-amine** (23.6 mg) was dissolved in dry deoxygenated DMF (0.3 mL) and added slowly to the activated diacid. The reaction was stirred overnight over argon and after 12 hours, the crude DMF solution was purified by injecting the solution on to an HPLC column where the final product **16** was readily purified (RP18, gradient, 90% $H_2O \rightarrow$ 100% acetonitrile). MS (ESI) m/z = 1199 ($M+H^+$). Other coumermycin analogs were prepared using an identical method.

BIOLOGICAL EVALUATION

To evaluate the effectiveness of the analogs of the present invention to act as chemical dimerizers of chimeric proteins, a BAF cell proliferation assay was performed.

BAF Cell Proliferation Assay

BAF cells are known in the art and are a murine cell line that is dependent on cytokine stimulation for growth. We have transfected BAF Cells with the human IL2 Receptor β chain. These transfected cells respond to human IL-2 by increasing their rate of cellular proliferation. This can be assayed by measuring the amount of tritiated thymidine that these cells incorporate into their DNA. One of the signaling proteins activated by IL-2 is the transcription factor STAT5b. This activation of STAT5b is responsible, in part, for the growth-promoting effects of IL-2.

STAT5b is activated by dimerization, an event that is dependent on its phosphorylation and which requires interaction of STAT5b with a ligand bound activated receptor. We can mimic this activation of STAT5b by transfecting BAF cells with a fusion protein consisting of the GyrB domain (the24 kDa amino-terminal fragment of bacterial DNA gyrase, amino acids 2-220) fused to the carboxy-terminus of STAT5b. Addition of the symmetrically dimeric antibiotic coumermycin artificially forces the dimerization of STAT5b-GyrB fusion proteins and results in an increased rate of cell growth that can be measured by the cell's ability to incorporate thymidine. This provides an assay to determine the ability of coumermycin analogs to promote dimerization of STAT5b-GyrB fusion proteins.

The assay is run as follows: BAF cells transfected with the human IL-2 receptor β chain and STAT5b-GyrB are washed twice in phosphate-buffered saline. They are then re-suspended in BAF cell media that lacks IL-2 and placed in a 96-well plate. Increasing amounts of coumermycin, or coumermycin analogs are then added to different wells in a 96-well plate and the cells are incubated overnight. Twenty-four hours later the cell media is spiked with tritiated thymidine and the cells are incubated for an additional 16 hours. The cells are subsequently harvested with a Wallac cell harvester and the amount of tritiated thymidine incorporated is measured using a β scintillation counter.

EXAMPLES

The following examples are for the purpose of illustration of the invention, and in no way are meant to limit or deter the scope of the invention. Those skilled in the art, after reviewing the examples, will appreciate other, various methods in which the invention can be practiced.

The abbreviations used in the examples are as follows:

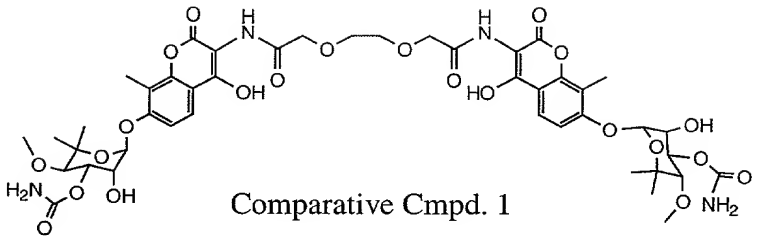
STAT = Signal Transducer and Activator of Transcription
IL = Interleukin as in Interleukin-2 (IL2)
PBS = Phosphate buffered saline
DMSO = Dimethyl Sulfoxide
BAF media

NEAA = Non-Essential Amino Acids
RPMI = cell growth media (Roswell Park Memorial Institute medium)
FCS = fetal calf serum
L-glu = the L form for glutamine
P/S = penicillin and streptomycin

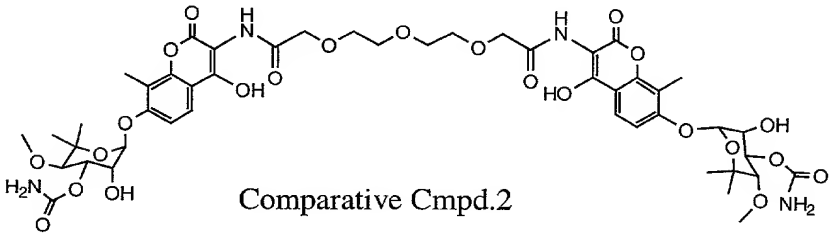
EXAMPLE 1

Compounds 5 and 6 were tested for BAF cell proliferation. Set up for BAF proliferation assay was as follows:

Spin down cells and wash one time in PBS. Resuspended in BAF cell media without cytokine at $2x10^5$ cells/ml. added 100 µl/well or $2x10^4$ cells. The compounds were prepared at 2 mg/ml (equivalent to 1.8 nM ±5%). Plates were set up so that each condition equaled 1 row of 12 (n=number of wells/row=12). DMSO being utilized as an initial control Compound 5 was prepared in concentrations of 1.6, 16, 163, 450, and 900 nM, and 1.8 µM in DMSO, and as a second control, IL-2 growth factor was added to coumermycin. Two novenamine anologs, Comparative Compounds (CCmpd) 1 and 2, below were also use in this example as control. It is known that novenamine analogs penetrate BAF cells poorly. The comparative novenamine compounds are as follows:



Comparative Cmpd. 1



Comparative Cmpd.2

Table 2 provides the concentration for the coumermycin control and compounds, as well as the proliferation results and number of wells/row (n) of the microplate.

Table 2

Proliferation of STAT5b-GyrB Transfected BAF Cells Following Stimulation with Coumermycin or Coumermycin Analogs

1	2	3	4	5	6	7	8	9	10
Stimulus	Conc.	Prolif.	S.D.	n	Stimulus	Conc.	Prolif.	S.D. 2	N
DMSO	0.1	15977	2549	12	DMSO	0.1	16215	1181	12
Cou. 16 nM	1.6	16137	1090	12	Cmpd 5 1.6 nM	1.6	15270	1157	12
Cou. 16.3 nM	16	16261	1342	12	Cmpd 5 16.3 nM	16	14277	1366	12
Cou. 163 nM	163	16836	3017	12	Cmpd 5 163 nM	163	14139	1182	12
Cou. 450 nM	450	18842	2046	12	Cmpd 5 450 nM	450	12732	1010	12
Cou. 900nM	900	21057	1239	11	Cmpd 5 900 nM	900	12315	941	12

Cou. 1.8 µM	1800	23199	1402	11	Cmpd 5 1.8 µM	1800	11751	960	12
IL-2		103613	7231	12	IL-2		104407	10989	12
DMSO	0.1	15971	1335	10	DMSO	0.1	17296	950	12
Cmpd 6 1.6 nM	1.6	14954	828	12	CCmpd 2 1.6 nM	1.6	14761	819	11
Cmpd 6 16.3 nM	16	14710	963	12	CCmpd 2 16.3 nM	16	14459	2101	12
Cmpd 6 163 nM	163	13351	884	12	CCmpd 2 163 nM	163	14516	1239	12
Cmpd 6 450 nM	450	12190	929	11	CCmpd 2 450 nM	450	13568	1428	12
Cmpd 6 900 nM	900	12872	890	12	CCmpd 2 900 nM	900	12281	630	12
Cmpd 6 1.8 µM	1800	10832	572	12	CCmpd 2 1.8 µM	1800	11676	1025	12
IL-2		108500	7069	12	IL-2		109880	5033	12
DMSO	0.1	16755	1269	12					
CCmpd 1 1.6 nM	1.6	15741	1283	12					
CCmpd 1 16.3 nM	16	14691	1354	12					
CCmpd 1 163 nM	163	13628	1796	12					
CCmpd 1 450 nM	450	12465	1108	12					
CCmpd 1 900 nM	900	11935	901	12					
CCmpd 1 1.8 µM	1800	11057	901	12					

IL-2		10461 6	4807	12					

EXAMPLE 2

In this example, Compounds 3, 4, 7, 8 and 9 were tested in BAF cell assays. The compounds were re-suspended in dimethylsulfoxide (DMSO) at a concentration of 2 mg/ml. Compounds 7 and 9 appeared slightly "purple" after dissolving in DMSO, perhaps a suggestion of some contamination with PNC-Amine. Next, the cells were washed 2 times with PBS. The re-suspended cells were present at a concentration of $1x10^5$ cells /ml in BAF media (RPMI, 10% FCS (fetal calf serum), 1% NEAA (non-essential amino acid), 1% L-G-lu, P/S and Hepes) without IL-2, added 100 µl to well (equal to $1x10^4$ cells/well).

The compounds were prepared in as follows:
DMSO was used as a control; each of the 5 compounds were combined with DMSO at concentrations of 1.8 µM, 900 nM, 450 nM, 90 nM 9 nM, and 900 nM. As a second control, coumermycin in IL-2 was utilized. Each solution at the aforementioned concentration condition, along with BAF cells, was placed in 1 row (12 wells) of a 96 well plate. Thereafter, 100 µL of compound (twice the concentration) was added to the wells. After 24 hours, 3-H thymidine was added to the wells, and the assays were harvested.

Table 3 provides the DMSO concentration for the coumermycin control and compounds, as well as the proliferation results and number of wells/row (n) of the microplate.

Table 3

Proliferation of STAT5b-GyrB Transfected BAF Cells Following Stimulation with Coumermycin or Coumermycin Analogs

1	2	3	4	5	6	7	8	9
Compound	Conc. (graph)	Prolif. (cpm	S.D.	N	Compound	Prolif. (cpm)	S.D. 2	N
DMSO	0.09	20912	1514	72	DMSO	20912	1514	72
Cou. 900 pM	0.9	18071	1690	12	Cmpd 8 90 pM	17973	1657	
Cou. 9 nM	9	20551	1903	12	Cmpd 8 9 nM	19377	1209	
Cou. 90 nM	90	25219	1159	12	Cmpd 8 900 nM	20715	1860	
Cou. 450 nM	450	27515	1907	12	Cmpd 8 450 nM	22936	1241	
Cou. 900 nM	900	30752	1743	12	Cmpd 8 900 nM	23858	1276	
Cou. 1.8 μM	1800	30664	1950	12	Cmpd 8 1.8μM	22772	1806	
IL-2		98745	7073	72	IL-2	98745	7073	72
DMSO	0.09	20912	1514	72	DMSO	20912	1514	72
Cmpd 9 900 nM	0.9	16826	1703		Cmpd 7 900 pM	16520	1628	
Cmpd 9 9 nM	9	18126	984		Cmpd 7 9nM	16599	1617	
Cmpd 9 90 nM	90	19177	1495		Cmpd 7 90 nM	17690	1562	

Cmpd 9 450 nM	450	20889	1588		Cmpd 7 450 nM	20553	1402	
Cmpd 9 900 nM	900	22210	2013		Cmpd 7 900 nM	20619	1563	
Cmpd 9 1.8 µM	1800	20367	1428		Cmpd 7 1.8µM	20207	1598	
IL-2		98745	7073	72	IL-2	98745	7073	72
DMSO	0.09	20912	1514	72	DMSO	20912	1514	72
Cmpd 3 900 pM	0.9	16313	1836		Cmpd 4 900 pM	20204	1825	
Cmpd 3 9 nM	9	16767	1622		Cmpd 4 9 nM	19290	2532	
Cmpd 3 90 nM	90	19920	1482		Cmpd 4 90 nM	19731	1856	
Cmpd 3 90 nM	450	22221	1967		Cmpd 4 450 nM	20726	1756	
Cmpd 3 900 nM	900	22824	2140		Cmpd 4 900 nM	22817	2099	
L880,078 1.8 µM	1800	21389	1761		Cmpd 4 1.8 µM	23135	1410	
IL-2		98745	7073	72	IL-2	98745	7073	72

EXAMPLE 3

A proliferation assay of Compound 1 using BAF cells transfected with the human growth factor IL-2RB chain and STAT5b-GyrB.

The assay was set up using DMSO; Compound 1 in DMSO concentrations of 900 pM, 9 nM, 90 nM, 450 nM, 900 nM, and 1.8 µM; and IL-2, as a control. Compound 1, in rows of 12, containing the aforementioned concentrations of

DMSO, coumermycin controls, and the cells were placed in wells of a microplate. After 24 hours 3H-thymidine at various concentrations was added to each well and allowed to proliferate.

Table 4 provides the DMSO concentration for the coumermycin control and compounds, as well as the proliferation results and number of wells/row (n) of the microplate.

Table 4

Proliferation of STAT5b-GyrB Transfected BAF Cells Following Stimulation with Coumermycin or Coumermycin Analogs

Conc.	Conc. (graph)	Cou. Prolif.	S.D.	n	Cmpd 1 Prolif	S.D.2	N
DMSO	0	8085	485	24	8085	485	24
900 pM	0.9	7422	299	12	7156	773	12
9 nM	9	8792	469	12	7653	409	12
90 nM	90	9860	319	12	7487	440	12
450 nM	450	10832	422	12	7209	292	12
900 nM	900	11470	750	12	7092	344	11
1.8 μM	1800	12308	618	12	6397	313	12
IL-2		31644	2005	24	31644	2005	24

EXAMPLE 4

A proliferation assay of Compound 2 using BAF cells transfected with the human growth factor IL-2Rß chain and STAT5b-GyrB.

The cells were placed in the wells at a concentration of $1x10^4$ cells/well (in 100 µL=$1x10^5$ cells/mL. The assay was set up using DMSO; Compound 2 in DMSO concentrations of 900 pM, 9 nM, 90 nM, 450 nM, 900 nM, and 1.8 µM; and IL-2, as a control. Compound 2, in rows of 12, containing the aforementioned concentrations of DMSO, coumermycin controls, and the BAF cells were placed in wells of a microplate. After 24 hours 3H-thymidine was added to each well and the cells were allowed to proliferate an additional 16 hours. Figure 12 provides a graphical illustration of the results of the proliferation.

An inhibition assay was also conducted for Compounds 1 and 2. For this assay, all wells contained a DMSO concentration of 90 nM of coumermycin. Compounds 1 and 2 were added at concentrations of 1.8 µM, 900 nM, 90 nM and 9 nM. The conditions of the assay were identical to those referenced early in this example, except that one-third of the amount of 3-H thymidine was used.

Tables 5 and 6 provide the concentration for the coumermycin control and compounds, as well as the proliferation results and number of wells/row (n) of the microplate.

Table 5

Proliferation of STAT5b-GyrB Transfected BAF Cells Following Stimulation with Coumermycin or Coumermycin Analogs

1	2	3	4	5	6	7	8	9
Compound	Conc. (graph)	Prolif. (cpm)	S.D.	n	Compound	Prolif. (cpm)	S.D. 2	N
DMSO	0.09	6161	424	21	DMSO	6161	424	21
Cou. 900 pM	0.9	6063	367	11	Cmpd 2 900 pM	6014	344	10
Cou. 9 nM	9	6750	674	11	Cmpd 2 9 nM	6031	326	12

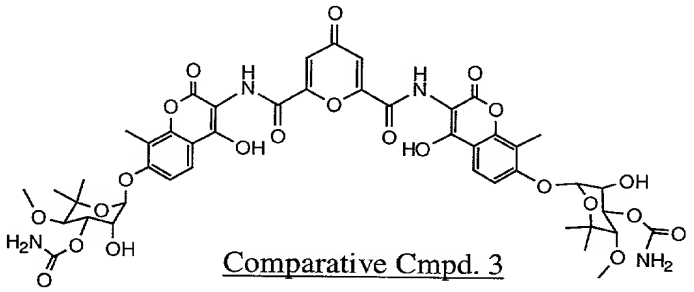
Cou. 90 nM	90	7595	437	10	Cmpd 2 90 nM	6623	241	11
Cou. 450 nM	450	8457	530	11	Cmpd 2 450 nM	7513	450	12
Cou. 900 nM	900	8636	607	11	Cmpd 2 900 nM	7798	463	11
Cou. 1.8 uM	1800	8610	553	11	Cmpd 2 1.8 uM	7825	343	12
IL-2		18285	681	12	IL-2	18285	681	12

Table 6

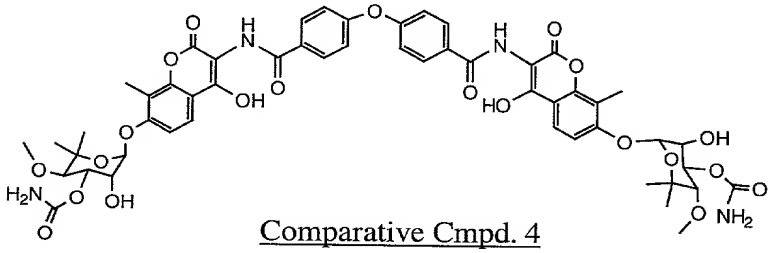
1	2	3	4	5	6
Compound	Inhibitor	Conc. (graph)	Prolif.	S.D.	n
Cou. 90 nM	DMSO	0.9	7595	437	10
Cou. 90 nM	Cmpd 1 9 nM	9	7713	328	12
Cou. 90 nM	Cmpd 1 90 nM	90	7683	297	11
Cou. 90 nM	Cmpd 1 900 nM	900	7597	209	11
Cou. 90 nM	Cmpd 1 1800 nM	1800	7271	273	10
Cou. 90 nM	DMSO	0.9	7595	437	10
Cou. 90 nM	Cmpd 2	9	7654	182	12
Cou. 90 nM	Cmpd 2	90	7316	391	11
Cou. 90 nM	Cmpd 2	900	8068	434	11
Cou. 90 nM	Cmpd 2	1800	8165	467	11

EXAMPLE 5

In this example, Compounds 1 and 2 were compared to 2 comparative novenamime compounds Comparative Compounds 3 and 4, for cell proliferation. The novenamime compounds are as follows:



Comparative Cmpd. 3



Comparative Cmpd. 4

The procedure for preparing the compounds, coumermycin controls, as well as the addition of BAF cells and 3-H thymidine were performed in accordance with the Example 4, above.

Table 7 provides the concentration for the coumermycin control and compounds, as well as the proliferation results and number of wells/row (n) of the microplate.

Table 7

Proliferation of STAT5b-GyrB Transfected BAF Cells Following Stimulation with Coumermycin or Coumermycin Analogs

1	2	3	4	5	6	7	8	9
Compound	Conc. (graph)	Prolif. (cpm	S.D.	n	Compound	Prolif. (cpm)	S.D. 2	N
DMSO	0.09	33562	2947	55	DMSO	33562	2947	55
Cou. 900 pM	0.9	36503	3292	12	Cmpd 2 900 pM	36052	2457	11
Cou. 9 nM	9	44455	4059	11	Cmpd 2 9 nM	38236	3693	12
Cou. 90 nM	90	51248	5116	12	Cmpd 2 90 nM	42674	3503	12
Cou. 450 nM	450	60293	5465	12	Cmpd 2 450 nM	47023	4085	12
Cou. 900 nM	900	64831	6507	12	Cmpd 2 900 nM	46989	3129	12
Cou 1.8 µM	1800	68728	3753	12	Cmpd 2 1.8 µM	46345	3344	12
IL-2		131314	9382	60	IL-2	131314	9382	60
DMSO	0.09	33562	2947	55	DMSO	33562	2947	55
CCmpd 4 900 pM	0.9	35624	3252	12	Cmpd 1 900 pM	31865	2146	12
CCmpd 4 9 nM	9	35985	4308	12	Cmpd 1 9 nM	34279	2795	12
CCmpd 4 90 nM	90	36764	2451	11	Cmpd 1 90 nM	33933	2752	12

CCmpd 4 450 nM	450	36763	3258	12	Cmpd 1 450 nM	35212	2613	12
CCmpd 4 900 nM	900	35379	3452	11	Cmpd 1 900 nM	33301	2530	12
CCmpd 4 1.8 µM	1800	31918	2788	11	Cmpd 1 1.8 µM	31029	2035	12
IL-2					IL-2	131314	9382	60
DMSO	0.09	33562	2947	55				
CCmpd 3 900 pM	0.9	32299	3045	12				
CCmpd 3 9 nM	9	33921	2985	12				
CCmpd 3 90 nM	90	33671	4001	11				
CCmpd 3 450 nM	450	33712	2205	12				
CCmpd 3 900 nM	900	31968	3032	12				
CCmpd 3 1.8 µM	1800	28434	1564	11				
IL-2		131314	9382	60				